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CATALYTIC EFFECTS ON THE LUMINESCENCE OF  
3-AMINOPHTHALHYDRAZIDE BY HEMINS  
AND HEMIN DERIVATIVES \*

(Received 16 December 1938)

[Following is a translation of an article by  
Otto Schales, Biochemical Institute of the  
University of Copenhagen, in the German-lang-  
uage periodical Berichte der deutschen che-  
mischen Gesellschaft (Reports of the German  
Chemical Society), Vol 72, No 1, 1939, pages  
167-177.]

As has been reported by H. O. Albrecht (1), the blue luminescence occurring during the oxidation of 3-aminophthalhydrazide (luminol) with hydrogen peroxide is catalytically intensified by manganese peroxide, colloidal platinum, blood, as well as potato peroxidase. However, the luminescence becomes particularly strong when a little hemin is added to the reaction mixture (2). Horseradish peroxidase is also effective according to R. Wegler (3); but it cannot bring about such a strong light emission phenomenon as hemin. H. Thielert and P. Pfeiffer (4) showed with the example of salicylaldehyde-ethylenediimine ferric chloride that also simpler iron complex salts than hemin can be catalytically effective. I have previously reported (5) about the effect of watersoluble c-hemin and some metal salts on luminescence; that report includes also a critical discussion of the forensic blood test with luminol which had been described by W. Specht (6).

The interest which exists for this intense light emission phenomenon from various points of view -- as a model for bioluminescence processes which are also connected with

\* The work described in this report was performed with the support of the Ella-Sachs-Plotz-Foundation and the van't Hoff fund.

an oxidation, as sensitive method for the detection of hydrogen peroxide and its formation in dehydration processes, and perhaps also as basis for a practical useful light source -- prompted me to study more carefully its course and the effects of various factors on it.

### 1. Effect of Hydrogen Ion Concentration

According to the literature, the system luminol- $H_2O_2$ -hemin exhibits luminescence only in an alkaline reaction; B. Tamamushi (7) has noted that a light emission occurs only at a pH above 12. This statement is not true. To be sure, the intensity of the luminescence is stronger in highly alkaline solutions than in weakly alkaline solutions, as R. Wegler (3) reported. In my experiments with buffered solutions there was also a decrease in luminescence with increasing hydrogen ion concentration and proportionately to this there was a color change in the light emission from blue to white. However, even at pH 5.10, a faint but nevertheless distinct luminescence can be observed.

### 2. Effect of Temperature

When two alkaline soda solutions of identical luminol and hemin concentrations are treated with the same amount of hydrogen peroxide -- one solution at 20 degrees and the other one at zero degrees, then the arising luminescences appear to be of equal intensity to the naked eye. However, this picture changes rapidly as is shown by Table 1:

Table 1

Time Change of Luminescence Intensity at  
Zero Degrees and at 20 Degrees

Temp.	Subjective impression of the intensity after						
	0	3	7	10	14	25	45 min.
0°	--	un- changed	still strongly blue	still distinct from 10m distance	--	still distinct from 5m distance	ex- tinct
20°	as at 0°	marked- ly wea- ker than at 0°	whi- tish	still de- tectable from 0.3m distance	ex- tinct	--	--

It is seen that the duration of the light emission is approximately three times longer at 0 degrees than at 20 degrees, which is quickly recognized with the fading, even by subjective observation. Actually, the product of intensity and the duration of luminescence should stay constant at different temperatures. At zero degrees a weaker and longer light emission should be expected. But it is understandable that the duration of the light emission at low temperature lasts longer in spite of the same initial intensity. Hemin decomposes in a side reaction part of the hydrogen peroxide. This side reaction is presumably inhibited by lower temperature (the main reaction seems considerably lessened); simultaneously the decomposing effect of the alkali (1 percent sodium carbonate solution) on  $H_2O_2$  slows down so that a larger amount remains for the light emission process itself. From the observation that a new addition of peroxide brings about new luminescence, it follows that the luminescence is stopped at the hydrogen ion concentrations chosen here on account of the  $H_2O_2$  consumption.

When mesohemin is used as catalyst instead of hemin under otherwise identical conditions, a considerably brighter luminescence is observed, which will be discussed later. The luminescence at 20 degrees lasts under the chosen conditions five minutes (hemin in a parallel experiment: 14 minutes); however at zero degrees the luminescence lasts 15 minutes, that is, also three times as long as at 20 degrees. After extinction, it is possible to induce new luminescence by fresh addition of  $H_2O_2$ .

### 3. Objective Measurement of the Luminescence Intensity

While our eye can detect well identical intensity of two emissions -- the use of colorimeters, step photometers or step-wise colored standard series is based on this capability of the eye -- the eye is not able to estimate numerically differences in intensity in a more or less satisfactory way. The comparison of the effect of various luminescence catalysts can therefore be accomplished only by objective measurements. In my experiments, this comparison was carried out with a selenium-photovoltaic cell coupled to a mirror galvanometer. To obtain comparable results, various factors have to be kept constant during the measurements; a 1 percent sodium carbonate solution was always used as solvent for the aminophthalic acid hydrazide and for the catalyst. Moreover, three parts of the 1 percent luminol solution were always mixed with three parts catalyst solution and subsequently with one part 0.03 percent hydrogen peroxide solution. The start of the addition of  $H_2O_2$  to the

luminol catalyst mixture was taken as the start of the luminescence.

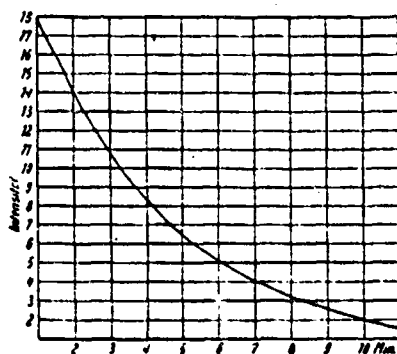


Fig. 1. Luminescence curve of the system luminol- $H_2O_2$ -hemin

The initial intensity which the catalyst can stimulate is not characteristic for the latter, but the entire course of the luminescence which is represented graphically by plotting the intensity along the ordinate (in centimeters of the galvanometer displacement) and the time along the abscissa. The first reading of the intensity is made one minute after the addition of  $H_2O_2$  for practical reasons. This method has, of course, the disadvantage that the initial luminescence can not be determined accurately for mixtures with a rapidly decreasing brightness. Furthermore, I try to designate the rate of intensity decrease by a term which I call luminescence half-time (HLD)  $\left[ \text{"Halbe-Luminescenz-Dauer"} \right]$ . With HLD I mean that period of time which passes until the intensity of the luminescence has reached half the value it had at the moment of the first reading. Fig. 1 shows the curve which I obtained when using hemin as catalyst. The HLD amounts to two minutes and 41 seconds. It should be added that the catalyst solution contained 26 milligram per liter chlorohemin and I must presuppose that the luminescence-catalytic activity of this solution had become constant.

#### 4. Activation of the "Hemin" Catalyst

We know from various studies, particularly those of R. Kuhn and L. Brann (8,9) as well as that of W. Langenbeck (10) that the catalytic and peroxidative activity of hemin may vary absolutely and with respect to one another when

small changes are introduced into the hemin molecule. I made the usual assumption that the luminescence-catalytic function of hemin is a peroxidative function and attempted to obtain more effective catalysts by preparing hemin derivatives in which the peroxidative activity is increased in comparison with that of hemin. Besides mesohemin -- which according to K. Zeile (11) is catalytically quite active in 0.1 normal NaOH while it is according to Kuhn and Brann (8) more strongly peroxidative in the alkaline region, but considerably weaker than hemin -- I studied a few parahemins whereby the following bases were used: pyridine, nicotine, 4(5)-methyl-imidazole, and p-methoxy-4(5)-phenylimidazole. W. Langenbeck, R. Hutschenreuter, and W. Rottig (12) showed that all these parahemins mentioned (with the exception of nicotinehemin which was not included in the studies) are (at pH 6-8) peroxidatively more effective than hemin. When the catalytic and peroxidative activity of hemin are both taken equal to one, then the following activities are calculated for the parahemins mentioned according to the data of these authors:

	peroxidase activity	catalase activity
hemin	1	1 (pH 8)
hemin pyridine	5	1.4 (pH 8)
hemin 4(5)-methylimidazole	7	4.5 (pH 8)
hemin 4(5)-phenylimidazole	14	1.1 (pH 7.5)
hemin p-methoxy-4(5)-phenylimidazole	15	0.3 (pH 7.5)

My experiments showed that pyridine and nicotine increase the luminescence-catalytic activity of hemin, cf Fig. 2. In connection with this, the HLD decreased which, in a series of experiments, was determined to be for pyridine-parahematin from 2 minutes 9 seconds to 2 minutes 25 seconds and for nicotine-parahematin from 1 minute 41 seconds to 1 minute 53 seconds. The effect of imidazole derivatives on the course of the luminescence is shown in Table 2. This table shows in each case two measurements, taken at random, which were carried out at different times with independently prepared catalyst solutions.

Methylimidazole causes still a distinct increase in hemin activity and the HLD decreases to 1 minute 38 seconds or 1 minute 40 seconds, respectively. Phenylimidazole weakens hemin considerably and decreases its HLD from 2 minutes 41 seconds to 1 minute 3 seconds or 1 minute 11 seconds,

respectively; finally, methoxy-phenylimidazole removes the luminescence-catalytic activity of hemin almost completely whereby the HLD is reduced to the range of 45 seconds to 1 minute.

Table 2  
Luminescence-Catalytic Activity of Some  
Imidazole-Parahematin

a) Zeit in Min.	b) Intensität d. Lumineszenz in cm Galvanometer-Ausschlag						
	c) Hämia	d) + Methylimidazol	e) + Phenylimidazol	f) + Methoxy-phenyl- imidazol			
1	17.9	24.0	23.3	3.7	3.1	0.6	0.6
1 1/2	16.0	19.9	18.6	2.6	2.3	0.5	0.4
2	14.0	15.3	15.1	1.9	1.7	0.3	0.2
2 1/2	12.2	12.5	12.3	1.3	1.3	0.2	0.1
3	10.7	10.5	10.3	1.0	1.1	0.1	0.0
3 1/2	9.4	8.9	8.7	0.7	0.75	0.0	
4	8.2	7.6	7.5	0.5	0.6		
4 1/2	7.2	6.3	6.4	0.35	0.45		
5	6.4	5.7	5.6	0.25	0.35		
5 1/2	5.7	5.0	4.9	0.2	0.25		
6	5.1	4.4	4.3	0.1	0.2		
6 1/2	4.5	3.9	3.8	0.0	0.15		
7	4.0	3.5	3.4		0.1		
7 1/2	3.5	3.1	3.0		0.05		
8	3.2	2.8	2.7		0.0		
8 1/2	2.8	2.5	2.4				
9	2.5	2.2	2.1				
9 1/2	2.3	1.9	1.9				
10	2.0	1.7	1.7				

[Legend:] a) time in min; b) intensity of the luminescence in cm galvanometer deflection; c) hemin; d) + methylimidazole; e) + phenylimidazole; f) + methoxyphenylimidazole.

Therefore, contrary to expectations, it turns out that the hemin derivatives phenylimidazole-parahematin and p-methoxy-phenylimidazole parahematin which others found to be particularly effective do not only bring about no increase in the luminescence intensity, but decrease the latter considerably and shorten simultaneously the luminescence period. The explanation of this statement is difficult, because it can hardly be assumed that the catalysts mentioned lose their peroxidative properties in 1 percent  $\text{Na}_2\text{CO}_3$



solution and become strong catalases. Moreover, mesohemin which is highly active catalatically in 0.1 normal NaOH solution (loc cit) increases the intensity of the luminescence considerably, cf Fig. 3.

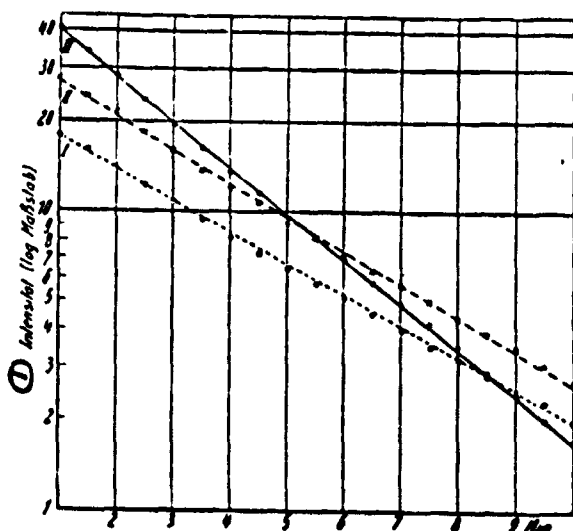


Fig. 2. Increase in luminescence-catalytic activity of hemin (I) by pyridine (II) and nicotine (III). (I) = intensity (logarithmic scale)

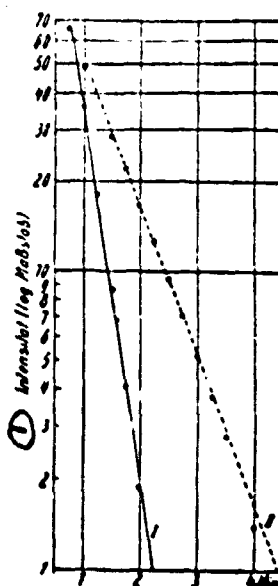


Fig. 3. Luminescence-catalytic activity of mesohemin (I) and chlorohemin (II). (I) = intensity (logarithmic scale)

This leads me to the assumption, as a temporary working hypothesis, that perhaps just these catalytic properties of the catalysts are the stimulating factors for luminescence. Such a thought is not as farfetched as it appears to be, because D. Keilin and E. F. Hartree (13) have found that the  $H_2O_2$  catalase formed during the dehydrating activity of xanthinioxidase is indispensable for the coupled oxidations of alcohols to aldehydes, although it should actually destroy the peroxide.

If my assumption is correct then those parahematin which possess an especially increased catalatical activity in comparison with hemin should be good luminescence catalysts.

K. G. Stern (4) has investigated the catalatic properties of a large number of hemin complexes. When the catalatic activity of hematin (at pH 7.3) is taken to be equal to one, then these values are calculated from his data on the activities of the parahematin with the following bases:

pyridine:	1.17 (pH 6.3)
l-histidine:	1.41 (pH 7.3 - 8.3)
methylimidazole:	1.44 (pH 7.9)
nicotine:	1.93 (pH 7.3)
histamine:	2.71 (pH 8.4)

(Compared with Langenbeck and coworkers (loc cit) it is apparent that here methylimidazole-parahematin does not differ so widely in its activity from hematin.)

It is essential that l-histidine, nicotine, and histamine activate the catalytic properties of hemin. My experiments showed now also an increase in the luminescence-catalytic activity of hematin by these bases (cf Table 3).

Table 3

Luminescence-Catalytic Activity of Histamine-Parahematin and l-Histidine-Parahematin

a.) Zeit in Min.	b.) Intensität der Lumineszenz in cm Galvanometer-Ausschlag				
	c.) Häm-in (Kontrolle)	d.) + Histamin		e.) + l-Histidin	
1	16.6	36.0	34.4	42.0	50.0
1 1/2	14.3	25.0	23.6	30.3	35.4
2	12.4	18.2	17.2	23.0	26.1
2 1/2	10.8	13.7	13.0	17.5	19.7
3	9.5	10.6	10.0	14.1	15.2
3 1/2	8.6	8.4	7.8	11.4	11.9
4	7.7	6.8	6.2	9.3	9.4
4 1/2	7.0	5.5	5.0	7.7	7.4
5	6.4	4.5	4.0	6.4	6.0
5 1/2	5.8	3.7	3.3	5.3	4.8
6	5.4	3.1	2.7	4.3	3.9
6 1/2	5.0	2.6	2.2	3.8	3.1
7	4.6	2.2	1.9	3.2	2.5
7 1/2	4.3	1.8	1.6	2.7	2.0
8	4.0	1.5	1.3	2.3	1.6
8 1/2	3.7	1.2	1.1	1.9	1.3
9	3.3	1.0	0.9	1.6	1.1
9 1/2	3.3	0.8	0.75	1.3	0.8
10	3.1	0.7	0.6	1.1	0.7
MLD	2' 40"	1' 1"	1' 0"	1' 11"	1' 3"

(Legend on following page)

[Legend:] a) time in min; b) intensity of the luminescence in cm galvanometer deflection; c) hemin (control); d) + histamine; e) + L-histidine.

In summary, it must be concluded from these activation experiments that some peroxidatively highly active parahematin are poor luminescence catalysts, while the parahematin with the highest catalytic activity bring about an increase in luminescence intensity.

Some remarks shall still be made about the course of the luminescence. The curve of Fig. 1, which is similar to the disintegration curve of radium, becomes a straight line when the intensity is plotted on a logarithmic scale (cf Fig. 2). However, this is only the case with hemin, nicotine-parahematin, pyridine-parahematin and phenylimidazole-parahematin; the other catalysts yield logarithmic curves which, in most cases, are composed of two straight lines of which the first one is steeper (up to approximately three minutes) (15). It is surprising, with the complex reaction mechanism which must be assumed for the occurrence of the luminescence that there are at all cases in which the decrease in light intensity follows the equation of a first order reaction and reflects presumably the monomolecular decomposition of  $H_2O_2$ .

I have tested the validity of the equation  $dx/dt = k(a - x)$  in the form  $1/t \cdot \ln(a/a-x) = k$  with the example of nicotine parahematin (whereby I substituted the natural logarithm by Brigg's logarithm) and calculate for:

nicotine-parahematin :  $k \times 10^3 = 1.55$  (largest deviation among 19 individual values + 4%),  
pyridine-parahematin :  $k \times 10^3 = 1.16$  (largest deviation among 19 individual values  $\pm 3.5\%$ ).

##### 5. Mesohemin, Chlorohemin and Hematin

When mesohemin is used as catalyst, the intensity of the luminescence is by far greater than that obtained with the catalysts discussed so far. Fig. 3 shows the course of the luminescence; it should be noted that the volume of the luminescent mixtures measured here is only 2/5 of the volume of the mixtures studied so far, i.e. the numerical values

read off from Fig. 3 must be multiplied by a factor of approximately 2.5 to become comparable with the data in the preceding tables and figs.

Surprisingly it turns out that chlorohemin has a similar, high activity when freshly used in a 1 percent sodium carbonate solution. The activity of the catalyst decreases apparently to the same extent to which chlorine is substituted by the hydroxyl group and reaches finally a constant limiting value. R. Kuhn and L. Brann (9) reported also previously about "aging phenomena" of hemin in weakly alkaline solutions which these authors explained with the substitution of the chlorine by a hydroxyl group. However, in their experiments, hematin had approximately the same activity as hemin, only the pH dependence of its activity was not as strong as that of hemin. The decrease in luminescence-catalytic activity of hemin occurs in a 10 percent  $\text{Na}_2\text{CO}_3$  solution considerably faster than in a 1 percent solution; Table 4 shows the changes in the latter case.

Table 4

Decrease of the Luminescence-Catalytic Activity of Chlorohemin During Storage in 1 percent  $\text{Na}_2\text{CO}_3$  Solution. (The volume of the reaction mixture amounts to 2/5 of the amount which had been measured in Figs 1 and 2 as well as in Tables 2 and 3.)

a) Zeit in Min.	b) . Intensität in cm Galvanometer-Ausschlag					
	c) 1 Stde.	d) 24 Stdn.	e) 2 Tage	f) 6 Tage	g) 14 Tage	h) 21 Tage nach Auf- lösen des Chlorhämins
1	49	44	36	20	10.6	7.0
2	16.7	16	17.8	13.3	7.3	5.4
HLD	40"	46"	59"	1' 40"	1' 45"	2' 25"

[Legend:] a) time in min; b) intensity in cm galvanometer deflection; c) 1 hour; d) 24 hours; e) 2 days; f) 6 days; g) 14 days; h) 21 days after dissolution of chlorohemin.

The activity of mesohemin remained unchanged after storage in a 1 percent  $\text{Na}_2\text{CO}_3$  solution for 14 days as far as the intensity 45 seconds after addition of  $\text{H}_2\text{O}_2$  is concerned, but the HLD decreased during this period from 13

seconds to 17.5 seconds, i.e. the reaction rate slows down somewhat.

#### 6. Hydrogen Peroxide Detection in 1 : 100 Million Dilution

W. Langenbeck and U. Ruge (16) determined and I could confirm their findings (5) that it is possible to detect  $H_2O_2$  by means of luminol in the presence of hemin as catalyst even at  $1:5 \times 10^6$  dilution. It is now possible to derive from my experience with the luminescence-catalytic activity of mesohemin and chlorohemin a more sensitive method for the detection of peroxide.

Reagent: One hundred milligram 5-aminophthalic acid hydrazide is dissolved in 100 cubic centimeter 1 percent  $Na_2CO_3$  solution and shortly before use of the reagent 3.0 milligram mesohemin or chlorohemin is added to this solution.

Use: Five cubic centimeter of the reagent are added, in a dark room, to 5 cubic centimeter of the solution to be tested for peroxide (test tube). Chlorohemin exhibits with the limiting hydrogen concentration of  $1 : 10^8$  still a weak light emission which, however, fades away rapidly; the mesohemin reagent, on the other hand, causes a strong light emission at the instant of the addition only after 10 seconds is this emission completely extinguished. Even with higher peroxide concentration, the mesohemin reagent is to be preferred because the luminescence caused by the reagent is, at the instant of its addition, always more intensive than that obtained using the chlorohemin-luminol mixture. Since the catalytic activity of mesohemin does not change when kept in a 1 percent  $Na_2CO_3$  solution (within 14 days), there exists, furthermore the advantage that the mesohemin reagent may be kept.

#### 7. Luminescence Catalysis by Salicylaldehyde-Ethylenediimine Ferric Chloride

H. Thielert and P. Pfeiffer (4) reported on the use of salicylaldehyde-ethylenediimine ferric chloride (SK) as catalyst for the luminescence of aminophthalic acid hydrazide and state that the intensity obtained by this catalyst is "approximately one third" of that obtained with hemin. They carry out their experiments in such a way that 3.5 milligram SK or 6 milligram hemin, respectively, are added to 10 cubic centimeter of Specht's luminol reagent. [Specht's reagent (6): to 0.1 gram luminol in a 100 cubic centimeter volumetric flask are added 50 cubic centimeter 10 percent

$\text{Na}_2\text{CO}_3$  solution as well as 15 cubic centimeter 3 percent  $\text{H}_2\text{O}_2$  and then filled up with water to the mark. Although Specht's reagent is not particularly useful for quantitative measurements because its peroxide content decreases continuously under the action of alkali, I have repeated Thielert and Pfeiffer's experiment exactly according to the data given by these authors. Table 5 shows the result.

Table 5

Comparison of the Activity of 6 Milligram Hemin and 3.5 Milligram SK per 10 Cubic Centimeter of Specht's Reagent

a) Zeit in Min.	b) Intensität d. Luminescenz		a) Zeit in Min.	b) Intensität d. Luminescenz	
	c) Hämín	SK.		c) Hämín	SK.
1	120	3.5	4	1.6	3.25
1 1/2	54	4.0	4 1/2	0.8	3.15
2	25	3.4	5	0.4	3.05
2 1/2	12	3.35	5 1/2	0.2	2.9
3	6	3.35	6	0.1	2.8
3 1/2	4.1	3.3	6 1/2	0.05	2.7
			7	0	2.65

Legend: a) time in min; b) intensity of the luminescence; c) hemin.

The intensity of the luminescence caused by SK is, thus, much weaker in the first minutes than one third of that caused by hemin, but the HLD reaches the high value in almost 19 minutes.

In further experiments that shall not be described in detail with this catalyst, it was found that soda solution deactivates the catalyst rapidly; this is superficially recognized by development of an orange color. The deactivation occurs almost quantitatively in 1 percent  $\text{Na}_2\text{CO}_3$  solution over a period of 24 hours.

Best results could be obtained with SK when it was suspended in a 1 percent  $\text{Na}_2\text{CO}_3$  solution, well shaken, and used quickly. When the concentration of catalyst, luminol, and  $\text{H}_2\text{O}_2$  are chosen, just as Thielert and Pfeiffer did, but the  $\text{Na}_2\text{CO}_3$  concentration is lowered to 1/5 of Thielert and

Pfeiffer's value, then the intensity of the luminescence is more than doubled. It is interesting that with this experimental change the catalyst must be present in the luminescent mixture for 3 minutes before its maximum activity is noted. Fig. 4 shows the course of the luminescence; simultaneously the decrease in activity on storage of the catalyst in soda solution may be seen.

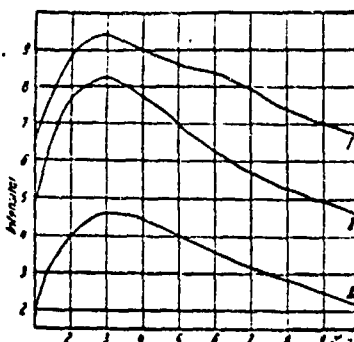


Fig. 4. Course of the luminescence with salicylaldehyde-ethylenediamine ferric chloride as catalyst (1/4 hour I; 3/4 hour II and 1 1/2 hour III after suspension of the catalyst in 1 percent  $\text{Na}_2\text{CO}_3$  solution).

#### 8. Luminescence Extinction by Higher Pyridine Concentrations

Some time ago, P. Holtz, and G. Triem (17), O. Schales (5), as well as C. Henze (18) have shown by means of luminol that during the action of oxygen on a number of compounds  $\text{H}_2\text{O}_2$  or unstable peroxides are formed. When these compounds react in the presence of oxygen with hydroxyhemoglobin and hemochromogens, green products are formed; the latter are called pseudohemoglobins or pseudochromogenes, respectively, [G. Barkan and O. Schales (19)]. Other authors who, likewise, considered the formation of such green blood color derivatives (20, 21) report on the difficulties which they encountered during detection of the thereby occurring formation of peroxide by means of luminol. Since the starting materials of these authors contained pyridine, the following has to be said. It is true that, when used in very small quantities, pyridine increases the luminescence intensity of

aminophthalic acid hydrazide, but in larger amounts it acts as luminescence inhibitor. When to 5 cubic centimeter of a 1 percent luminol solution (in 1 percent sodium carbonate solution) in a test tube 5 cubic centimeter of a  $4 \times 10^{-5}$  M hemin solution (in 1 percent sodium carbonate solution) and 0.2 cubic centimeter 3 percent  $H_2O_2$  are added, a strong and lasting luminescence occurs. If now pyridine is added dropwise, the luminescence becomes at first stronger, but further addition of pyridine weakens it. After addition of 2 cubic centimeter pyridine, the previously bright luminescence has faded and is hardly recognizable. Since considerably smaller amounts of peroxide occur during formation of the pseudochromogenes than has been admitted in the cited experiment, it must be assumed that the intensity of the luminescence has been weakened by the presence of pyridine to such an extent that it can no longer be detected.

#### Description of the Experiments

1. Materials: Luminol was prepared following the procedure described by E. H. Huntress, L. N. Stanley and A. S. Parker (22). The substituted imidazoles were prepared according to R. Weidenhagen and R. Herrmann (23) and mesohemin according to J. Zaleski (24) from mesoporphyrin according to H. Fischer, E. Bartholomaeus and H. Rose (25). The hydrogen peroxide solutions were always freshly prepared by diluting Perhydrol (Merck).

2. Luminescence at Different pH Values: For these experiments the chlorohemin was first dissolved in the alkaline component of the buffer (NaOH in case of a citrate buffer and tertiary phosphate in case of phosphate buffers) and then more diluted with buffer solution until a solution of the desired pH was obtained, which contained 1 milligram chlorohemin in 10 cubic centimeter. For instance, 2 cubic centimeter 0.1 normal NaOH containing 1 milligram chlorohemin was diluted with another 2 cubic centimeter 0.1 normal NaOH and 6 cubic centimeter citrate; the pH of the mixture is 5.91. The same procedure was used for the preparation of 0.1 percent solutions of luminol in a buffer. Ten cubic centimeter buffered luminol solution and 5 cubic centimeter 3 percent  $H_2O_2$  were added to 10 cubic centimeter hemin-buffer.

3. Luminescence at Zero Degrees and at Twenty Degrees: Three cubic centimeter of a  $4 \times 10^{-5}$  molar hemin solution in 1 percent  $Na_2CO_3$  solution and 3 cubic centimeter of a 0.1 percent luminol solution in 1 percent  $Na_2CO_3$  solution



were put into each of two test tubes. One of the test tubes was put into melting ice. Into each of two other test tubes was put 1 cubic centimeter 0.03 percent  $\text{H}_2\text{O}_2$  solution. At the same time the luminol-hemin solutions were decanted into each of the two test tubes containing  $\text{H}_2\text{O}_2$  and the cooled hemin solution was again put into ice. See Table 1 for the course of the luminescence.

4. Objective Intensity Measurements: The photocell was in a light-proof box. The distance between the scale and the mirror galvanometer was 2 meter. Petri dishes of 71 millimeter diameter were used as containers for the luminescent mixtures in all quantitative measurements. Fifteen [6] cubic centimeter 0.1 percent luminol solution in 1 percent  $\text{Na}_2\text{CO}_3$  was put into the Petri dish followed by 15 [6] cubic centimeter of  $4 \times 10^{-5}$  molar hemin solution in 1 percent  $\text{Na}_2\text{CO}_3$  solution (prepared from a ten times more concentrated standard solution in 10 percent  $\text{Na}_2\text{CO}_3$  solution by dilution with water; however, in experiments with chlorohemin, section 5., these solutions were obtained by direct weighing into a 1 percent  $\text{Na}_2\text{CO}_3$  solution) and finally 5 [2] cubic centimeter 0.03 percent  $\text{H}_2\text{O}_2$  solution. The volumes given in brackets refer to Fig. 3 and Table 4. The stop watch was put into action at the instant of the  $\text{H}_2\text{O}_2$  addition. The mixture was subsequently well stirred and the measurements were started after the galvanometer oscillations had stopped.

5. Experiments with Paranematin: These catalyst solutions were obtained in such a way that to  $1 \times 10^{-3}$  mole of the corresponding bases was added 10 cubic centimeter of the  $4 \times 10^{-4}$  molar hemin solution in 10 percent  $\text{Na}_2\text{CO}_3$  solution. If necessary, the mixture was heated on a water bath until the base was mostly dissolved and finally made with water to a volume of 100 cubic centimeter. Fifteen cubic centimeter of the catalyst solution was taken for each measurement and mixed with luminol and  $\text{H}_2\text{O}_2$  as described above.

6. Experiments with Salicylaldehyde-ethylenediamine Ferric Chloride: The experiments given in Table 5 were carried out with hemin and SK according to the procedure of Thielert and Pfeiffer. For the measurements represented in Fig. 4, the following procedure was used. To 35 milligram SK in a glass-stoppered flask was added 50 cubic centimeter of a solution containing 100 milligram luminol and 500 milligram  $\text{Na}_2\text{CO}_3$ . The mixture was well shaken and 5 cubic centimeter of the suspension was transferred into a Petri dish by means of a pipet. To this 5 cubic centimeter of a 0.9 percent

H<sub>2</sub>O<sub>2</sub> solution was added. The changes in luminol and H<sub>2</sub>O<sub>2</sub> concentrations, compared with the experiments using the other catalysts, were made to conform with Thielert and Pfeiffer.

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